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## **Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase**

Dechant, Reinhard ; Binda, Matteo ; Lee, Sung Sik ; Pelet, Serge ; Winderickx, Joris ; Peter, Matthias

**Abstract:** Glucose is the preferred carbon source for most cell types and a major determinant of cell growth. In yeast and certain mammalian cells, glucose activates the cAMP-dependent protein kinase A (PKA), but the mechanisms of PKA activation remain unknown. Here, we identify cytosolic pH as a second messenger for glucose that mediates activation of the PKA pathway in yeast. We find that cytosolic pH is rapidly and reversibly regulated by glucose metabolism and identify the vacuolar ATPase (V-ATPase), a proton pump required for the acidification of vacuoles, as a sensor of cytosolic pH. V-ATPase assembly is regulated by cytosolic pH and is required for full activation of the PKA pathway in response to glucose, suggesting that it mediates, at least in part, the pH signal to PKA. Finally, V-ATPase is also regulated by glucose in the Min6 -cell line and contributes to PKA activation and insulin secretion. Thus, these data suggest a novel and potentially conserved glucose-sensing pathway and identify a mechanism how cytosolic pH can act as a signal to promote cell growth.

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# **The N-terminal domain of the V-ATPase subunit 'a' is regulated by pH *in vitro* and *in vivo***

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**Abstract:**

Regulation of the activity of vacuolar ATPase (V-ATPase) is a well known, yet poorly understood phenomenon, which might underlie the contribution of V-ATPases in various cellular signaling processes<sup>1</sup>. In yeast, V-ATPase is regulated by glucose and contributes to activation of cAMP-dependent protein kinase A (PKA). We have recently shown that, *in vivo*, glucose regulates V-ATPase through cytosolic pH, suggesting that V-ATPase contains a pH sensitive subunit, which regulates assembly of the holo-complex<sup>2</sup>. Here, we present the purification and biochemical characterization of the N-terminal domain of subunit 'a', Vph1N, which has been suggested to act as a pH sensor in mammalian cells<sup>3</sup>. Interestingly, our studies demonstrate pH-dependent oligomerization of this domain *in vivo* and *in vitro*. Moreover, we identify a membrane proximal region that is required for the pH-dependent oligomerization, and suggest a speculative model for the regulation of the V-ATPase holo-complex by pH.

## Introduction:

The vacuolar ATPases (V-ATPase) are highly conserved proton pumps, embedded in membranes of organelles of biosynthetic and endocytic pathways, thereby mediating intra-luminal acidification. V-ATPase activity is required for vacuolar protein turnover, vesicular trafficking and vacuolar fusion<sup>1, 4</sup>. In addition, recent findings indicate a role of V-ATPases in signal transduction in multiple systems<sup>2, 5-7</sup>.

V-ATPases consist of a membrane bound  $V_0$  sector (the proton channel) and the reversibly attached  $V_1$  sector, which is responsible for ATP hydrolysis. Regulation of V-ATPase often affects the assembly state of the holo-complex. For example, V-ATPase assembly is regulated by glucose in yeast and mammalian cells<sup>8, 9</sup>. Various kinases have been implicated in the regulation of V-ATPase activity, but the underlying mechanism of regulation remains unclear<sup>8, 10-15</sup>.

Interestingly, V-ATPase has been shown to interact with a downstream effector, the Rab-GEF ARNO, in an activity-dependent manner<sup>16</sup>. Based on this work, it has been suggested that V-ATPases themselves act as pH sensors<sup>3</sup>. Consistent with this idea, we have recently shown that, *in vivo*, V-ATPase assembly is regulated by cytosolic pH. Importantly, cytosolic pH is regulated by glucose levels, thereby identifying a molecular mechanism for V-ATPase regulation by glucose<sup>2</sup>.

More specifically, regulation and localization of V-ATPase is mediated by subunit ' $a$ '<sup>2, 17</sup>. Structural data indicate that this subunit consists of an N-terminal cytoplasmic domain, which is connected by a short linker to the membrane-bound C-terminal domain (Figure 1A and B, <sup>18</sup>). Moreover, a major

structural rearrangement of this subunit accompanies V-ATPase disassembly<sup>19</sup>, raising the possibility that pH-dependent regulation of this domain might mediate regulation of the V-ATPase holo-complex in response to glucose (Figure 1A, <sup>2</sup>).

## Results

To obtain biochemical evidence supporting this hypothesis, we aimed to purify and characterize the N-terminal domain of Vph1p, which encodes the vacuolar isoform of subunit 'a' in yeast. Therefore, we recombinantly expressed a fragment containing the first 418 amino acids, (Vph1N, Figure 1B), which includes the entire cytoplasmic domain of Vph1p, but stops before the first transmembrane domain. In addition, we sought to address a potential role of the linker region, connecting the cytoplasmic and membrane-bound domains in the pH-dependent assembly of V-ATPase. Secondary structure predictions revealed a possible alpha helix at the C-terminal end of the expressed protein fragment (data not shown), which might be part of the flexible linker region observed in EM. Therefore, we also expressed a truncated form of Vph1N, which contains only amino acids 1-385, Vph1N $\Delta$ L, and thus eliminates the predicted helix.

Expression of these constructs as N-terminal fusions to Maltose binding protein (MBP) yielded sufficient amounts of proteins with good purity using a one-step affinity purification (Figure 1C). Interestingly, reproducible purification was dependent on the use of phosphate buffer with high buffering capacity, already indicating a pH sensitivity of the protein.

CD-spectra of the purified MBP fusion protein confirmed proper folding with mostly alpha helical properties (Figure 1D), which was

consistent with previous observations and secondary structure predictions (<sup>20</sup> and data not shown). Recording of CD-spectra in buffers at different pH values did not reveal any detectable differences (Figure 1D), demonstrating that pH does not lead to major changes in the secondary structure of Vph1N.

However, when characterized in gel-filtration experiments, Vph1N eluted in two major peaks from Sepharose 6 columns, suggesting that Vph1N forms homo-oligomers *in vitro* (Figure 2A). Estimation of the sizes of these peaks using size standards was most consistent with the elution of monomers and octameric complexes. Moreover, incubation of the protein in buffers with lower pH significantly increased the octameric fraction (Figure 2A and B), demonstrating pH-dependent oligomerization of Vph1N *in vitro*.

Surprisingly, the truncated protein, Vph1N $\Delta$ L, mostly eluted as monomeric protein under all conditions tested (Figure 2A), suggesting that the predicted helix is required for oligomerization and possibly contributes to its pH regulation.

To examine whether Vph1N is also regulated by pH *in vivo*, we expressed the same fragments in yeast and followed pH-dependent oligomerization in total cell extracts. As shown in Figure 1C, Vph1N was detected in two major peaks following gel filtration, and decreasing pH shifted the ratio towards the high molecular weight form. In contrast, no significant differences in the elution profile were observed for Vph1N $\Delta$ L at different pH, thus recapitulating the data obtained with the recombinant proteins *in vitro*. Therefore, we conclude that Vph1N undergoes pH-dependent regulation *in vitro* and *in vivo*, strongly supporting the notion that

the N-terminal domain of subunit 'a' mediates pH-dependent regulation of V-ATPase assembly.

## Discussion

In an attempt to further understand pH regulation of the V-ATPase holo-complex, we sought to biochemically characterize the cytoplasmic domain of the V-ATPase subunit 'a' from yeast. Indeed, we observed a pH-dependent oligomerization of the purified Vph1N domain *in vitro*, and after overexpression *in vivo*.

Our data confirm and extend a previously published analysis of the mammalian a2 subunit<sup>20</sup>. Similar to our study, Merkulova and co-workers<sup>20</sup> found that the mammalian a2 subunit consists of mostly alpha helical fold and elutes in multiple peaks from a gel-filtration column, consistent with oligomerization of this domain. Strikingly, the protein was stabilized at high pH, indicating similar pH-dependent properties. However, the mammalian a2 fragment was stabilized in the presence of detergents and interacted with liposomes, thereby displaying properties of a membrane-bound protein. In contrast, Vph1N could be purified in the absence of detergents in solutions with high buffer capacity as an MBP fusion. It is likely that MBP acts as a solubility tag, overcoming the hydrophobicity of the domain. Indeed, Vph1N was largely insoluble as a His-tagged protein under the same conditions (data not shown).

pH-dependent regulation of this domain could readily explain the regulation of V-ATPase assembly in response to glucose. While our studies indicate oligomerization of Vph1N, it remains to be examined if regulated oligomerization indeed contributes to V-ATPase assembly *in vivo*. Structural

EM studies have only indicated one 'a'-subunit per V-ATPase holo-complex, thereby arguing against oligomerization within a single V-ATPase holo-complex *in cis*. Thus, homotypic interaction between cytosolic domains of subunit 'a' would have to occur *in trans*, linking two or more V-ATPase complexes.

An alternative explanation might be provided by structural predictions of Vph1N, which we have obtained by *ab initio* structure predictions. While Vph1N and Vma5p, which encodes the 'C' subunit of V-ATPase, do not share significant sequence similarity at the primary sequence level, Vph1N could be readily modeled based on the published crystal structure of Vma5p (Figure 2D, <sup>21</sup>) using an alignment based on secondary structure predictions (<http://meta.bioinfo.pl>). Vma5p reversibly associates with both the V0 and the V1 sector upon V-ATPase assembly<sup>22</sup>. Interestingly, it directly interacts with Vph1p based on crosslinking experiments<sup>23</sup>. Thus, *in vivo*, Vph1N and Vma5p might form hetero-dimers based on a topologically similar interaction that can lead to homo-oligomerization of Vph1N *in vitro*. It will therefore be interesting to further characterize binding of Vph1N and Vma5p both *in vivo* and *in vitro* and determine if regulation of this interaction might contribute to the assembly of the V-ATPase holo-complex.

In any case, identifying the pH sensitive residues that mediate the pH-dependent regulation of Vph1N will be crucial to further elucidate the mechanism of V-ATPase regulation. Based on the pKa values for free amino-acids in solution, only histidine residues are likely to change the protonation state under physiological conditions<sup>3</sup>. However, pKa values for the side-chains of amino acids are strongly dependent on their local environment within the protein, thereby complicating predictions of potential candidates



based on primary sequence. For example, regulated protonation of aspartate side-chains has been shown to mediate the regulation of pH-sensitive ion-channels<sup>24</sup>.

Recently, we have screened for mutations in Vph1p that alter pH-sensitive assembly of V-ATPase *in vivo*<sup>2</sup>. Interestingly, we identified a single substitution, D329N that affected V-ATPase disassembly upon starvation. Unfortunately, using our *in vitro* assay for pH-sensitive oligomerization of Vph1N, we did not observe any change in the pH sensitivity of the mutant protein (data not shown), suggesting that protonation of this residue is not regulating V-ATPase assembly *in vivo*. Rather, D329 might be required to transduce a conformational change upon protonation within Vph1N to mediate V-ATPase holo-complex assembly *in vivo*. Consistent with this finding, *vph1-D329N* mutations only delayed, but did not abolish V-ATPase regulation by glucose<sup>2</sup>. Conversely, as C-terminal truncation of Vph1N prevents oligomerization *in vitro* and *in vivo*, residues within the very C-terminus of Vph1N might be good candidates for future studies. Ultimately however, determination of the Vph1N structure will be crucial to resolve the molecular details of pH-dependent regulation of V-ATPase assembly.

## Materials and Methods

### *Protein expression and gel-filtration*

Proteins were expressed as N-terminal MBP fusions in BL21\* cells. Cells were lysed in 125 mM Na-PO<sub>4</sub> buffer (pH 7.4) supplemented with 2 mM DTT, 5% Glycerol, and 2mM EDTA. Fusion proteins were purified using Amylose beads (NEB), eluted with maltose and dialyzed into PBS (10 mM Na-PO<sub>4</sub>, 150 mM NaCl, pH 7.4) supplemented with 20% glycerol and 2mM DTT. After purification, aliquots of the protein were adjusted to different pH by addition of Na-phosphate and incubated at 30°C for 30 min to allow for equilibration of oligomers. Gel filtration was performed using a Superose 6 column (Amersham) and proteins were detected by UV absorption. Size calibration of the column was performed using commercial size standards (Amersham) and peaks were integrated using ImageJ software (<http://rsb.info.nih.gov/ij/>). CD-spectroscopy was performed on a Jasco J-710 (OmniLab) instrument.

Yeast extracts were prepared in 125 mM Na-PO<sub>4</sub> buffer (pH 6.0 or pH 7.2), 10% glycerol, 0.2% NP-40, 5 mM MgCl<sub>2</sub>, 2 mM DTT and cleared by ultracentrifugation (48.000 g, 20 min). Approximately 4 mg extracts were separated on a Superose 6 column (Amersham) and analyzed by western-blotting using polyclonal HA-antibodies (Abcam).

### *Vph1N structure prediction*

The structure prediction of Vph1N was generated using the BioInfo Metaserver (<http://meta.bioinfo.pl>) and MODELLER software<sup>25</sup>. Protein

structures were visualized in MacPyMol (The PyMOL Molecular Graphics System, Schrödinger, LLC).

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**Table 1: Plasmids used in this study**

Plasmid No	Genotype	Source
pMALc2	<i>MBP</i>	NEB
pRD6	<i>MBP-VPH1N</i> (1-418)	This study
pRD7	<i>MBP-VPH1NΔL</i> (1-385)	This study
pRD8	<i>pRS416-ADH-HA3-VPH1N</i> (1-418)	This study
pRD9	<i>pRS416-ADH-HA3-VPH1N</i> (1-385)	This study

**Figure legends****Figure 1: Recombinant expression of the N-terminal domain of V-ATPase subunit ‘a’.**

(A) Model for pH regulation of V-ATPase by subunit ‘a’ (indicated in green) and (B) schematic representation of the domain structure of subunit ‘a’, VPH1. L: linker domain. Fragments expressed as MBP- or HA-fusions are indicated. (C) Aliquots of the purified proteins were separated by SDS-PAGE and stained using coomassie to assess purity of the samples. (D) CD-spectra of Vph1N recorded after equilibration to pH 7.4 (high pH) and pH 6.0 (low pH). The mean molar ellipticity ( $\Theta$ ) is plotted as a function of the wavelength.

**Figure 2: Vph1N oligomerizes in a pH-dependent manner *in vitro* and *in vivo***

(A) Representative chromatograms of three independent experiments of gel filtration experiments of Vph1N (left panel) and Vph1NΔL (right panel) adjusted to the indicated pH. Elution volumes and molecular mass of proteins (in kD) used as size standards are above the chromatograms. (B) The octamer-to-monomer ratio of purified Vph1N was quantified and plotted as a function of pH. (C) Representative western blots of three independent experiments of selected fractions from gel-filtration experiments with yeast strains expressing

the indicated constructs. Extracts were prepared with buffer of indicated pH.

(D) Structural model of the Vph1N fragment (aa 57-365) derived by *ab initio* structure predictions using the published structure of Vma5p (pdb accession 1U7L) as a template for modeling.

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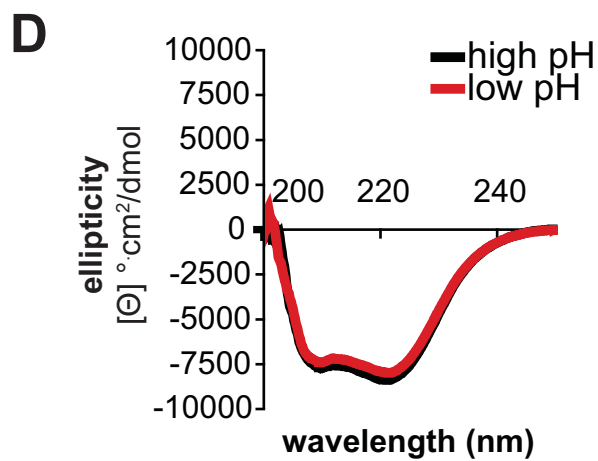
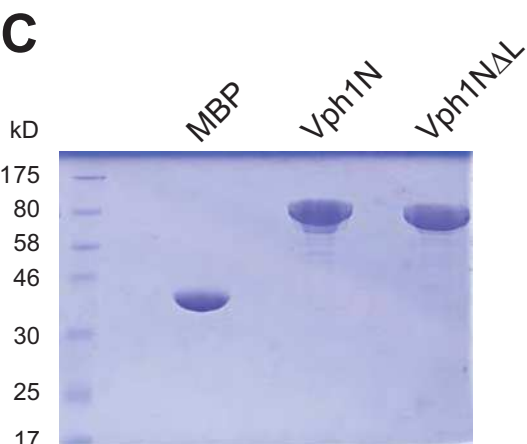
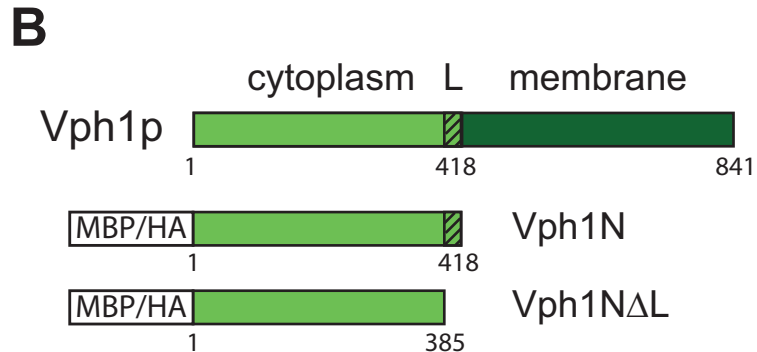
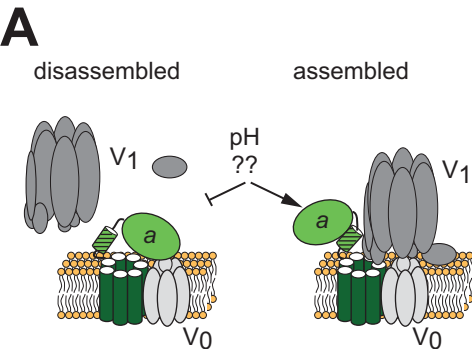
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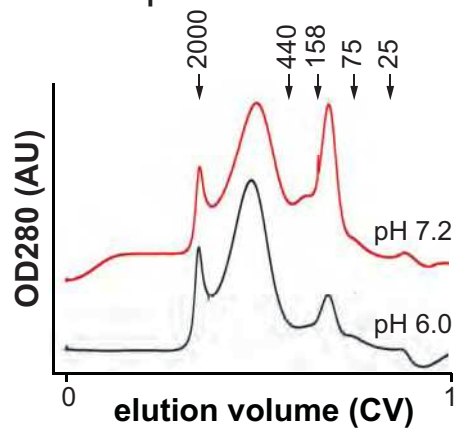
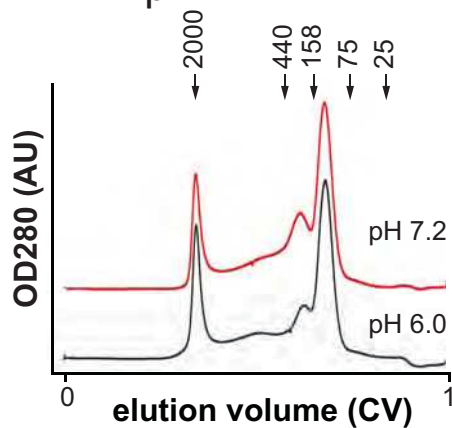
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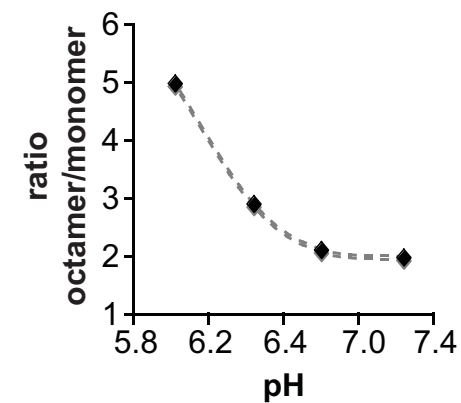


**A**

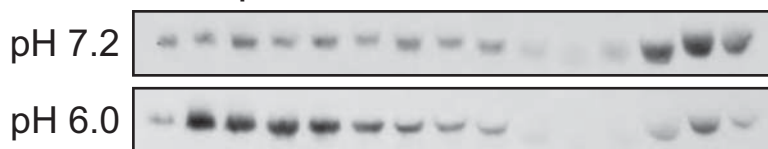
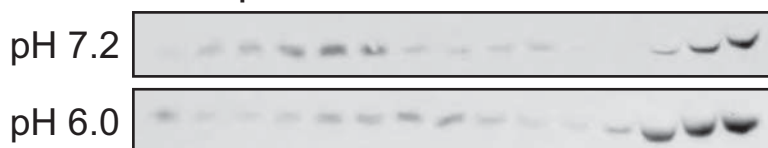
MBP-Vph1N

MBP-Vph1N $\Delta$ L**B**

MBP-Vph1N

**C**

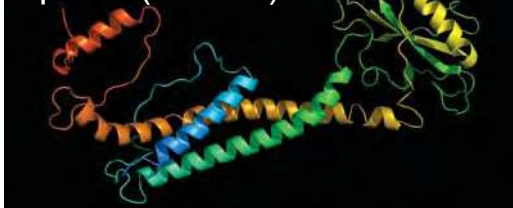
HA-Vph1N

HA-Vph1N $\Delta$ L

elution volume

**D**

Vph1N (57-365)



Vma5

